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Rapid Communication

Combination of nitrate (N, O) and boron isotopic ratios with microbiological indicators for the determination of nitrate sources in karstic groundwater

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Environmental context. Nitrate contamination of drinking water quality may be critical, particularly in rural areas where agricultural practices may release large amounts of nitrogen. Knowledge of the source of such contamination, mandatory for water supply management, can be successfully acquired by combining the natural stable isotopes of nitrate, boron isotopic ratios and microbiological indicators.

Abstract. A new approach based on measurements of nitrate and boron isotopic composition associated with microbiological indicators for the determination of nitrate origin in karstic groundwater (SW, France) is presented. Nitrate and boron isotopic data indicate an animal source of nitrate ($\delta^{15}N$–$NO_3^- > 5$ ‰, $\delta^{18}O$–$NO_3^- < 10$ ‰ and $\delta^{11}B$ ~25 ‰). Microorganism detection (bacteriophages) confirmed contamination from animal sources and proved fast water transfer (2–3 days) from surface to groundwater.

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Nitrate produced by anthropogenic activities is an important environmental issue, particularly in agricultural regions and especially when water is used as drinking water. Determination of the source of nitrate among the many potential sources is the first step towards water quality improvement.

Recent studies have proven the efficiency of the coupled use of nitrate (N and O) and boron isotope ratios to identify the origin of nitrate in water.[1–3] Nitrate from mineral fertilisers is characterised by atmospheric values of $\delta^{15}N$ (~0 ‰) and $\delta^{18}O$ (~23.5 ‰), whereas nitrate derived from organic fertilisers, livestock effluents and sewage is usually enriched in ^{15}N ($\delta^{15}N$ varies from +8 up to +20 ‰) because of ammonium volatilisation.[4–8] As animal and domestic waste $\delta^{15}N$ overlap, additional tracers such as boron are used to discriminate between these two sources.[3]

Boron is a co-migrant of nitrate and a tracer of domestic water input to the environment.[9] Furthermore, it is not affected by processes affecting nitrate (e.g. denitrification).[3] Although fertiliser $\delta^{11}B$ exhibits a wide range of values (−10 to 60 ‰), these ratios are significantly different from sewage (−2 to +2 ‰) and manure contaminations (5–50 ‰).[1–3,9]

In addition to this dual isotopic approach, we tested microbiological indicators (bacteriophages and *Bacteroidales*) to characterise the origin of faecal contamination occurring in groundwater.[10–14] F-specific RNA bacteriophages (FRNAPHs) are viruses, infecting bacteria of the gastrointestinal flora, with genogroups associated with animal (GI and GIV) or human (GII and GIII) faeces.[10–12,15] *Bacteroidales* is an order of Bacteria present in high numbers in the gastrointestinal tract of warm-blooded animals with host-specific distribution (humans, ruminants and pigs).[13,14,16] The use of microbiological markers allows a better discrimination among organic contaminants and gives complementary information on the transfer times within an aquifer (with a lifetime lasting a few days in the environment).

This new multidisciplinary approach was used to determine the origin of increasing nitrate concentrations in karstic groundwater located in an agricultural area. The borehole studied (F1) was dug up to 100 m deep in a Cretaceous limestone outcropping at the top of an anticline (Fig. 1). It is used for drinking water production for the South Aquitaine region (SW France), but nitrate concentrations have steadily increased from 5 mg L^{-1} in the 1970s to 25 mg L^{-1} since 2004 and traces of faecal contaminants have frequently been detected in the groundwater. The limestone aquifer is fed by deep groundwater but also by local recharge in the carbonate outcropping area (Fig. 1). The karstic nature of limestone potentially favours fast communication from surface to groundwater.

Water samples were regularly collected from this borehole from October 2010 to October 2012 at the pump outlet.

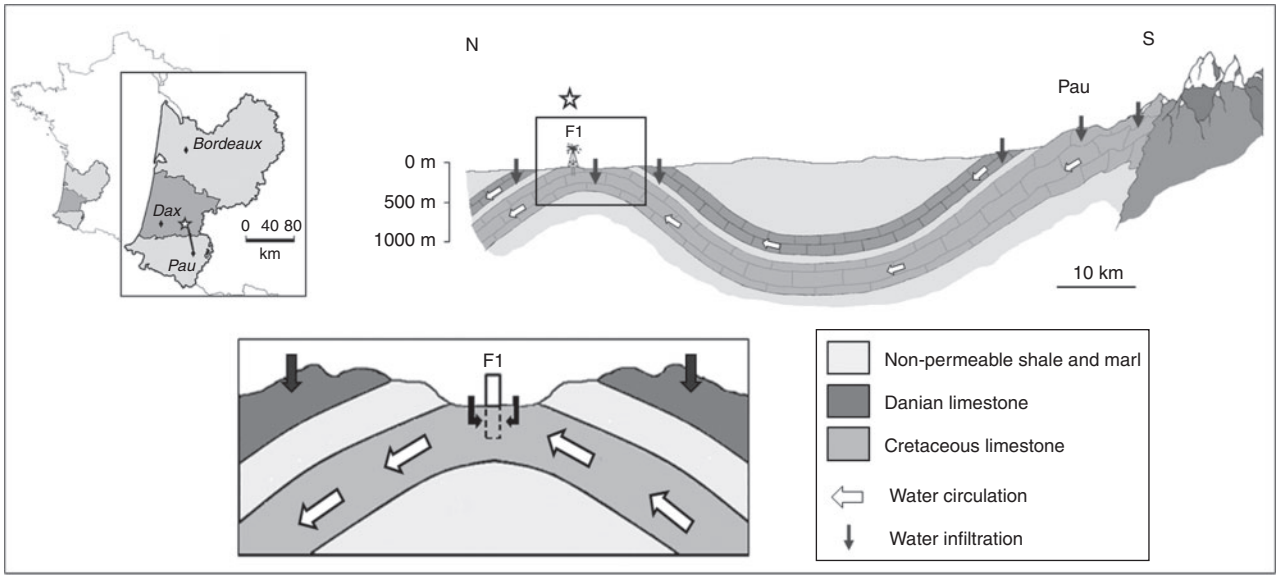

Fig. 1. Geographic location of the F1 borehole (white star) and geologic structure of the anticline. Magnification of the F1 borehole and the conceptual alimentation model.

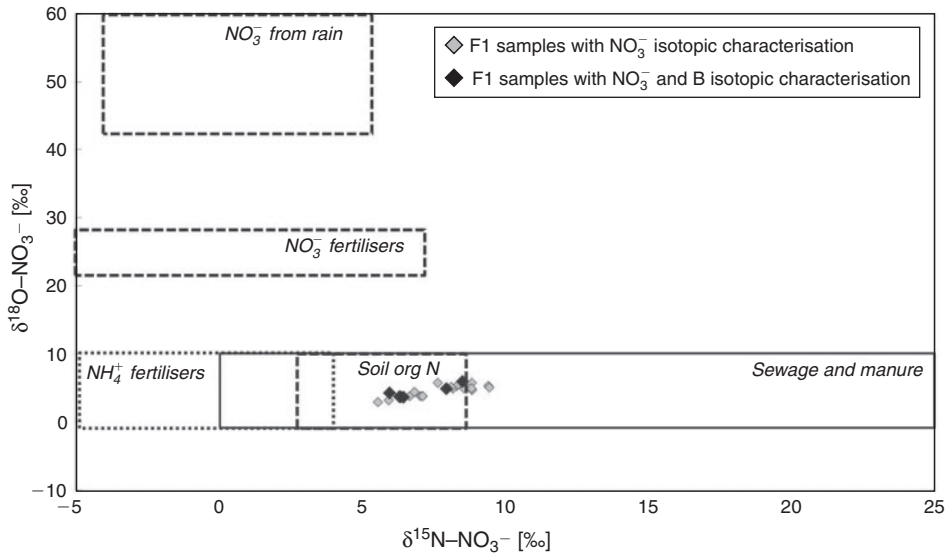

Fig. 2. Nitrate isotopic values of F1 water and comparison with those noted in the literature as potential sources of nitrate.[1–3]

Multilevel sampling was carried out twice (February and September 2011) using a mini pump within the borehole. All the samples were filtered through a 0.45-µm nylon membrane.

For nitrate concentrations, samples were stored frozen and were measured by high-performance liquid chromatography (HPLC Dionex, AS12 column). For the characterisation of the N and O isotopic composition of nitrate, samples were poisoned with $HgCl_2$ and analysed after reduction to nitrite through a granular cadmium-filled column. Nitrite was then converted into nitrous oxide by adding azide.[17] The determination of $\delta^{15}N$ and $\delta^{18}O$ of N_2O was performed through a purge-and-trap and continuous-flow isotope ratio mass spectrometry system (DeltaVplus Thermo coupled with Gas Bench II). The method was calibrated with nitrate standards (USGS-32, $\delta^{15}N = 180\,‰$, $\delta^{18}O = 25.7\,‰$; USGS-34, $\delta^{15}N = 1.8\,‰$, $\delta^{18}O = 27.9\,‰$ and USGS-35 $\delta^{15}N = 2.7\,‰$, $\delta^{18}O = 57.5\,‰$). The linearity of the analysis was checked with an internal nitrate standard (IAEA, $\delta^{15}N = 4.7\,‰$, $\delta^{18}O = 25.6\,‰$). The precision was 1.2 ‰ for $\delta^{15}N$ and 1.3 ‰ for $\delta^{18}O$.

For boron analyses, samples were acidified to pH 2 with HNO_3. Boron concentrations were determined by inductively coupled plasma optical–atomic emission spectroscopy (ICP OES/AES) JY2000. The $^{11}B/^{10}B$ isotopic ratios were measured by a multicollector inductively coupled plasma mass spectrometer (Neptune, ThermoScientific) using a direct-injection nebuliser (d-DIHEN, Analab).[18] Boron was first extracted from the samples using ion exchange chromatography. A volume of 5–30 mL of the samples at pH 9 was introduced into a column filled with 50 µL of resin (Amberlite IRA-743). Boron was retained on the resin and then eluted with dilute

Table 1. Nitrate and boron concentrations and associated isotopic compositions

F1, samples collected at the pump outlet; F1-*x* m, sample collected at a depth of *x* m as indicated. CFU, colony forming units; Inv. flora, Invasive flora; PFU, plaque forming unit

Sample name	Sampling date	$[NO_3^-]$	$\delta^{15}N$	$\delta^{18}O$	[B]	$\delta^{11}B$	Aerobic mesophilic flora		Total coliform	Standard fecal indicator		Bacteriophages	
							36 °C	22 °C		*E. coli*	Enterococci	Total	Genogroup G1
		($mg\,L^{-1}$)	(‰)	(‰)	($\mu g\,L^{-1}$)	(‰)	(CFU mL^{-1})	(CFU mL^{-1})	(CFU/100 mL)	(CFU/100 mL)	(CFU/100 mL)	(PFU/3 L)	(PFU/3L)
F1	19-Oct-10	23.9	5.9	3.3	–	–	–	–	–	–	–	–	–
F1	11-Jan-11	25.0	6.7	3.9	9.4	25.0	–	–	–	–	–	–	–
F1-30 m	15-Feb-11	25.2	6.3	3.8	10.9	25.1	29	23	4	<1	<1	3	3
F1-41 m	15-Feb-11	24.9	6.3	3.8	11.2	25.7	18	19	10	<1	<1	1	1
F1-52 m	15-Feb-11	23.4	5.6	3.0	–	–	40	29	10	<1	1	11	11
F1-73 m	15-Feb-11	22.4	7.0	3.9	–	–	50	35	11	<1	<1	9	8
F1-85 m	15-Feb-11	19.4	6.0	4.4	9.3	25.0	161	282	570	<1	<1	10	10
F1	09-Mar-11	25.0	7.9	5.0	11.3	25.4	–	–	–	–	–	–	–
F1	05-Apr-11	24.0	8.5	6.1	10.6	25.2	16	118	1	<1	<1	<1	–
F1	24-May-11	23.1	7.6	5.9	–	–	Inv.flora	18	1	<1	<1	<1	–
F1	25-May-11	23.5	7.1	3.9	–	–	<1	12	<1	<1	<1	<1	–
F1	26-May-11	22.6	6.8	4.5	10.7	25.6	8	25	6	<1	<1	<1	–
F1	27-May-11	24.0	8.3	5.4	–	–	–	–	–	–	–	<1	–
F1	21-Jun-11	23.9	6.4	3.8	–	–	–	–	–	–	–	–	–
F1	12-Aug-11	20.5	8.0	5.1	–	–	–	–	–	–	–	–	–
F1-30 m	19-Sep-11	23.7	8.2	5.0	–	–	Inv.flora	59	<1	<1	1	<1	–
F1-41 m	19-Sep-11	23.5	8.6	4.8	–	–	Inv.flora	37	2	2	1	<1	–
F1-52 m	19-Sep-11	22.8	8.8	4.8	–	–	14	45	5	4	1	<1	–
F1-73 m	19-Sep-11	21.4	8.8	5.9	–	–	65	230	3	3	1	<1	–
F1-85 m	19-Sep-11	19.6	8.1	5.9	–	–	107	154	<1	2	1	<1	–
F1	28-Oct-11	22.0	8.8	5.3	–	–	–	–	–	–	–	–	–
F1	19-Jan-12	24.0	9.4	5.2	–	–	–	–	–	–	–	–	–
F1	21-Aug-12	24.4	7.1	4.2	–	–	30	110	5	1	2	<1	–
F1	23-Oct-12	22.1	–	–	–	–	–	–	4	4	4	<1	–

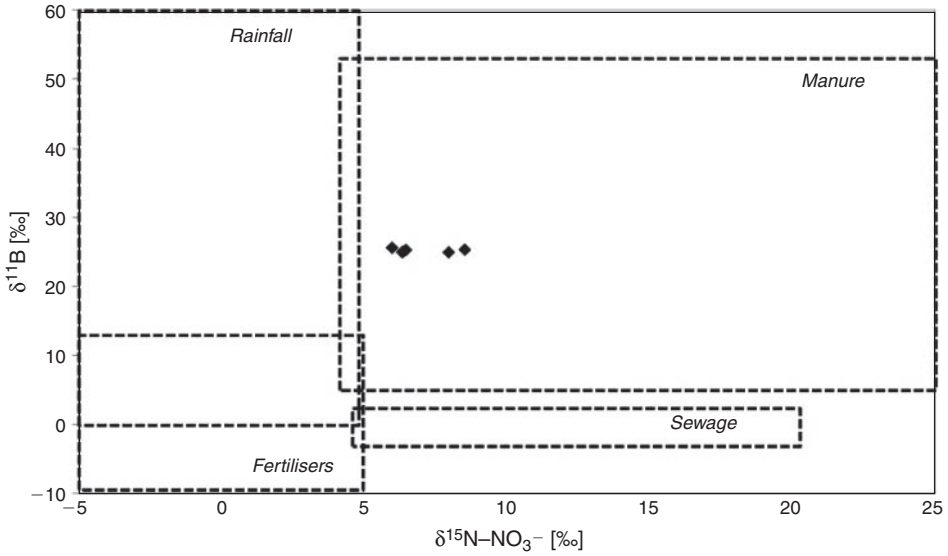

Fig. 3. Relationship between $\delta^{15}N–NO_3^-$ and $\delta^{11}B$ of F1 water and comparison with those noted in the literature as potential sources of nitrate and its co-migrant boron.[4–6]

HNO_3 (0.1 and 0.5 N).[18,19] $\delta^{11}B$ values were calculated by bracketing sample measurements with NBS-951 boron standard measurements. The external reproducibility of $\delta^{11}B$ measurements for natural water samples was 0.25 ‰ (2 s.d.).

For microbiological analyses, specific sampling bottles with sodium thiosulfates were used. Standardised methods were used to determine the concentration of total coliforms and *Escherichia coli, Enterococcus* species and total aerobic mesophilic flora.[20–22] FRNAPHs were enumerated by concentrating 3 L of a water sample using the membrane filtration–elution method.[23] Infectious FRNAPHs were counted (double agar-layer technique).[24] FRNAPHs contained in one plaque were collected, re-suspended in 1 mL of phosphate buffered saline (PBS) with 15 % glycerol and stored at −20 °C until genotyping, which was performed using a one-step real-time reverse-transcription polymerase chain reaction (RT-qPCR) kit (Quanti-Tech Probe RT–PCR, Qiagen) and previously described primers and probes.[11] To search for *Bacteroidales*, 2 L of sample water were filtered through a 0.22-µm pore size polycarbonate membrane. The filter was immersed in a guanidinium thiocyanate (GITC) lysis solution and stored at −80 °C until DNA extraction with a Qiamp DNA minikit (Qiagen). Previously described primers and probes were used to quantify ruminant (Rum-2-Bac),[14] pig (Pig-2)[13] and human (HF183)[16] *Bacteroidales* markers. Standard curves were calculated for plasmids containing the target sequence. The presence of PCR inhibitor factors was monitored by adding a known plasmid concentration to the sample. The results were expressed as several copies in 100 mL of water. PCR reactions were performed in duplicate for each sample with a Rotor gene 6000 thermocycler.

Nitrate concentrations measured in the borehole varied from 19.4 to 25.2 mg L^{-1}. The lowest values (19.4 and 19.6 mg L^{-1}) correspond to the deepest samples (85 m) from February and September 2011. This is consistent with the conceptual model of a double alimentation of the borehole: deep and slightly contaminated water and nitrate-contaminated surface water with a rapid infiltration rate.

The $\delta^{15}N$ and $\delta^{18}O$ ranges usually reported in the literature for the different potential sources of nitrate are presented in Fig. 2 with our isotopic data for the groundwater samples. Our $\delta^{15}N$ and $\delta^{18}O$ data fall within the organic boxes (sewage, manure or soil organic nitrogen-derived nitrate) with respective values of 5.6–9.4 and 3.0–6.1 ‰ (Table 1, Fig. 2).

Boron concentrations and isotopic compositions were measured for six samples, chosen to be representative of temporal and spatial variations. B concentrations varied only slightly, with an average value of 10.6 ± 0.7 µg L^{-1}. The $\delta^{11}B$ of these samples are rather homogeneous with an average value of 25.3 ± 0.3 ‰ (Table 1, Fig. 3). Combined $\delta^{15}N$ and $\delta^{11}B$ (Fig. 3) data correspond to values usually reported for nitrate originating from an animal source. It excludes nitrate derived from domestic effluents. This could be derived from manure spreading on maize fields or from localised livestock effluents.

The occurrence of the total viable count in the water of the F1 borehole ranged between <1 and 282 CFU mL^{-1} (colony-forming units per millilitre). Low coliform bacteria concentrations were observed in most water samples. However, standard faecal indicators (*E. coli* and *Enterococcus*) were detected in only three campaigns – September 2011, August 2012 and October 2012 – with concentrations under 4 CFU per 100 mL. FRNAPHs were detected in February 2011 at different depths of the F1 borehole with 3, 1, 11, 9 and 10 plaque forming units (PFU per 3 L) at respective 30-, 41-, 52-, 73- and 85-m depths. These concentrations are actually the same order of magnitude and cannot be considered significantly different for the five depths. Genotyping methods concluded that all these phages belong to genogroup I, which is generally associated with animal faecal pollution. Nevertheless, more extensive FRNAPH genotyping would be necessary to corroborate these preliminary results. Moreover, none of the host-specific *Bacteroidales* markers (human, porcine or ruminant) were detected, suggesting that another animal effluent could be responsible for faecal contamination occurring in the spring (duck and chicken farming are dominant in the studied area). Furthermore, the very short life span of microorganisms in groundwater (<2 days for bacteriophages)[25] proves that transfer from the surface to groundwater could be very fast, possibly favoured by karstic channels.

The determination of the origin of nitrate is an essential preliminary step for water resource management and remediation. In agricultural areas, where there can be multiple nitrate

sources, the combination of nitrate and boron isotopic composition can be very helpful to differentiate the different sources. Moreover, in the case of faecal contamination, and when groundwater potentially receives direct infiltration from the surface, characterisation of microorganisms provides valuable information on the source of the contamination.

This new approach was applied here to the case of a karstic spring where nitrate concentrations have gradually increased over the last 40 years and where episodic faecal contaminations are measured.

The combined approaches indicate that borehole F1 water is contaminated with nitrate from animal effluents. The existence of rapid surface water transfer due to the karstic nature of the aquifer allows the detection of these specific microorganisms.

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